Engineered Human Skin Model Using Poly(ADP-Ribose) Polymerase Antisense Expression Shows a Reduced Response to DNA Damage

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Poly(ADP-ribose) polymerase (PADPRP) modifies nuclear proteins in response to DNA-damaging agents. The principal organ subject to exposure to many of these agents is the skin. To understand the role of PADPRP in the maintenance of the epidermis, a model system has been developed in which we have selectively lowered the levels of this enzyme by the use of induced expression of antisense RNA. Human keratinocyte lines were stably transfected with the cDNA for human PADPRP in the antisense orientation under an inducible promoter. Induction of this antisense RNA in cultured cells selectively lowers the levels of PADPRP mRNA, protein, and enzyme activity. Induction of antisense RNA also led to a reduction in the levels of PADPRP in individual cell nuclei, as well as the loss of the ability of cells to synthesize and modify proteins by poly(ADP-ribose) polymer in response to DNA damage.

When keratinocyte clones containing the antisense construct or empty vector alone were grafted onto nude mice, they formed histologically normal human skin. The PADPRP antisense construct was also inducible *in vivo* by the topical application of dexamethasone to the reconstituted epidermis. In addition, poly(ADP-ribose) polymer could be induced and detected *in vivo* following the topical application of a DNA alkylating agent to the grafted transfected skin layers. Accordingly, a model system has been developed in which the levels of PADPRP can be selectively manipulated in human keratinocytes in cell culture, and potentially in reconstituted epidermis as well. This system will be a useful tool to study the role of PADPRP and DNA repair in general in essential biologic processes in the epidermis. *Key words: grafting/MNNG/sulfur mustard/DNA repair. J Invest Dermatol 105:38–43, 1995*

Poly(ADP-ribose) polymerase (PADPRP) is a nuclear enzyme that binds to and is activated by single- and double-stranded DNA breaks. The active enzyme catalyzes the poly(ADP-ribosyl)ation of certain nuclear proteins in the vicinity of the damaged DNA, using nuclear nicotinamide adenine dinucleotide (NAD^+) as a substrate [1]. Nuclear acceptor proteins for PADPRP include histones, high-mobility group chromosomal proteins, topoisomerases [2], protein ICP4 of herpes virus [3], and SV40 large T antigen [4–6]. PADPRP also undergoes automodification.

PADPRP can be activated by a number of agents, including chemical carcinogens, as well as by ultraviolet and ionizing radiation. Thus, PADPRP is likely to play an important role in the repair of DNA within the epidermis. The localization of PADPRP to the lower layers of the epidermis including the basal or proliferating cells, which are more susceptible to the effects of DNA damaging agents, is consistent with this notion [7]. The role of PADPRP in mediating the biologic response to DNA strand breaks is also supported by a number of studies that show that the increase in PADPRP activity may be responsible for or contribute to the blistering response of individuals exposed to sulfur mustards, and possibly to other DNA strand-breaking agents [8–12]. In addition to its significance in DNA repair, numerous studies have shown a role for PADPRP in cellular proliferation and differentiation [6,13].

Most studies supporting a function for PADPRP in these processes have relied on the use of chemical inhibitors of the enzyme, many of which lack specificity [14]. We recently established a method to selectively lower levels of PADPRP by the use of constructs that express antisense transcripts to the PADPRP cDNA [15]. DNA repair in response to strand-breaking agents was delayed, active gene repair was reduced, and there was increased amplification of genes coding for selectable markers [15–17].

Differentiation has been also shown to be associated with alterations in the expression of PADPRP, and inhibition of PADPRP can modulate the program of terminal differentiation [18–20]. We have shown that in 3T3 L1 preadipocyte mouse cells, the induction of antisense RNA directed against mouse PADPRP cDNA results in the inhibition of differentiation of cells into mature adipocytes [21].

To examine the role of PADPRP in differentiation and DNA repair in the epidermis, we were interested in developing a system to selectively lower the levels of PADPRP in cultured human

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Abbreviations: MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; NAD^+, nicotinamide adenine dinucleotide; PADPRP, poly(ADP-ribose) polymerase.

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keratinocytes, and in reconstituted epidermis. To our knowledge, this is the first report of a genetically engineered graft system in which endogenous levels of a keratinocyte enzyme can be inducibly altered *in vivo*. This system would be an important tool to study the role of PADPRP in essential biologic processes in the epidermis.

MATERIALS AND METHODS

Cell Culture, Transfections, and Grafting HPV 18 E6/E7–immortalized human keratinocytes were a kind gift from R. Schlegel [22]. Keratinocytes were grown in keratinocyte growth medium plus Dulbecco's modified Eagle's medium (3:1) containing 2.5% fetal bovine serum. Cells were transfected by lipofection reagent (Life Technologies, Inc.), and G418-resistant colonies were isolated. 5×10^6 human keratinocytes were grafted along with 8×10^6 primary mouse fibroblasts onto nude mice using the grafting-chamber technique as previously described for mouse keratinocytes [23].

Plasmids and Polymerase Chain Reaction (PCR) Analysis pMX18, containing the human cDNA encoding PADPRP in the antisense orientation in pMAMneo (Promega), has been described [15].

For PCR, cell extracts containing total genomic DNA were derived from each clone by methods described previously [24]. The upstream amplimer was the MMTV LTR sequence: ACA GTG GCT GGA CTA ATA GAA C. The downstream amplimer was located within the DNA-binding region of human PADPRP: GTT AGA ATG TCT GCC TTA CTG GT. Forty PCR cycles were performed on a thermal cycler (Thermolyne), with a denaturing step at 95°C for 15 seconds, annealing at 58°C for 30 seconds, and primer extension at 72°C for 30 seconds. This was followed by a 4-min extension step at 72°C. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide.

Antibodies and Immunocytochemical Analysis Antibody to poly-(ADP-ribose) was derived as described previously [7]. Rabbit antisera against human PADPRP was a kind gift from R. Roeder.

Cells were grown on 8-well chamber slides (Tissue-Tech). The MMTV-driven constructs were induced by the addition of 1 μM dexamethasone to the culture medium. After different time intervals, cells were washed twice in phosphate-buffered saline, and fixed by the addition of 10% ice-cold trichloroacetic acid. After 10 min, cells were washed successively with 70%, 90%, and absolute ethanol for 10 min. Slides were then processed for immunofluorescence. Immunofluorescence was performed as described [25] with anti-human PADPRP antibody diluted 1:500 and anti-PADPRP diluted 1:1000. For keratins, mouse monoclonal antibody was used (Chemicon). Immunofluorescence was visualized using a Zeiss immunofluorescent microscope equipped with a Nikon camera.

Western Blotting Control or antisense cells were lysed in 5% sodium dodecylsulfate (SDS); 20% β-meracaptoethanol; 0.125 M Tris-Cl, pH 6.8, and proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. PADPRP was visualized with rabbit antisera and electrochemiluminescence (ECL; Amersham).

Ribonuclease Protection Assay and *In Situ* Hybridization Total RNA was obtained for each cell clone [26] and analyzed by the ribonuclease protection assay [27]. The PADPRP-radiolabeled RNA probe used to detect the endogenous PADPRP mRNA was derived from the full-length human PADPRP cDNA in pGEM 4Z (Promega) transcribed *in vitro* with T7 polymerase, yielding a 618-bp probe protecting a 588-bp fragment representing the 3′ region of PADPRP mRNA. As an internal control, RPAs contained a radiolabeled RNA probe derived from a riboprobe vector containing a 220-bp Pst I fragment of the P_0 human acidic ribosomal protein [28], because the level of this mRNA is relatively invariant [29].

For *in situ* hybridization, graft sites were treated topically with 75 μg dexamethasone in acetone, and quick-frozen on dry ice. *In situ* hybridization was performed as described previously [30]. A riboprobe derived from the full-length human PADPRP cDNA was made as described [15].

RESULTS

We examined a number of different cell lines for their ability to undergo normal differentiation in the graft system, because few immortal keratinocyte lines have been described that retain the ability to undergo normal differentiation [31]. Based upon the results of the grafting experiments (below), we elected to use a clone of human epidermal keratinocytes immortalized with the E6 and E7 genes of HPV 18, which formed a histologically and immunocytochemically normal skin. These cells were transfected with human PADPRP cDNA in the antisense orientation under the control of the MMTV promoter **(Fig 1*A*)**. Approximately 25 G418-resistant clones were isolated. To assay stable integration, PCR analysis was performed. Twenty of the clones were positive for the correct integration of the PADPRP cDNA with respect to the MMTV promoter, as determined by the presence of a specific 771-bp amplified fragment detected on ethidium bromide–stained agarose gels **(Fig 1*B*)**. Two stable lines (AS-1 and AS-2) containing the antisense construct were chosen for further analysis, along with control cells transfected with the vector alone.

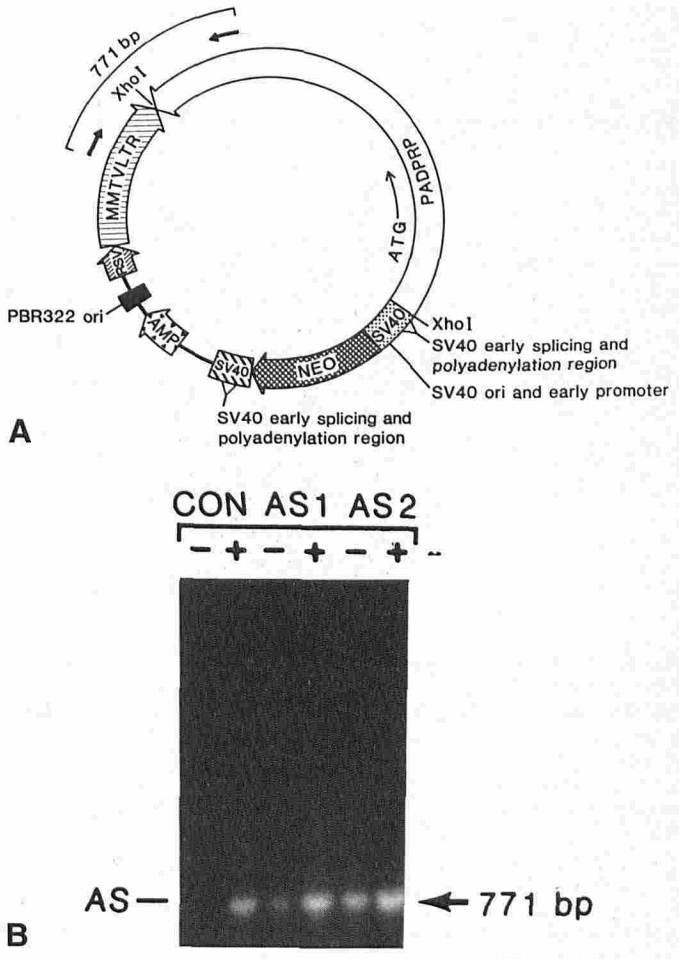

Figure 1. *A*) Structure of pMAMneo containing PADPRP antisense cDNA, and location of PCR amplimers. The expression vector contains the glucocorticoid inducible MMTV promoter ligated to an inverted orientation of human PADPRP cDNA comprising the entire PADPRP untranslated and translated regions as described previously [15]. The PADPRP sequence is flanked downstream by the SV40 early splicing and polyadenylation region and the neomycin (neo) and ampicillin (amp) resistance genes. The entire cloned plasmid is 12.2 kb. Amplimers used for PCR analysis are shown as *black arrows*. *B*) PCR analysis of the inverted orientation of the integrated PADPRP cDNA in genomic DNA. Cells transfected with either pMAMneo (control cells; *lanes 1–2*) or transfected with the antisense construct (*lanes 4–6*), were subjected to PCR analysis using amplimers described in *Materials and Methods*. The presence of the 771-bp fragment in DNA derived from two independently derived antisense clones (AS1 and AS2) indicates the integration of the antisense construct, as well as the correct orientation of the PADPRP cDNA with respect to the MMTV promoter. As a control for efficiency of the PCR reaction, genomic DNA was spiked with 1 pg of plasmid used for the transfection (+).

Endogenous PADPRP mRNA is Depleted in Transfected Cells We determined the fate of the PADPRP mRNA following induction of PADPRP antisense RNA. From previous studies, we have found that PADPRP mRNA is relatively unstable. Therefore, a sensitive ribonuclease protection assay was employed to deter-

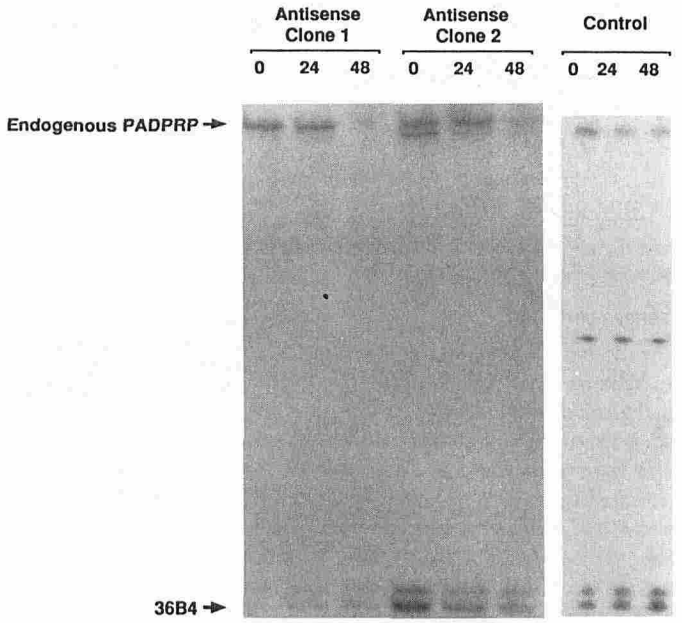

Figure 2. PADPRP antisense induction lowers endogenous PADPRP mRNA. Cultures were incubated in the presence of 1 μM dexamethasone for the indicated time periods (in hours). Total RNA was isolated and analyzed by a ribonuclease protection assay, using the PADPRP mRNA-specific probe (*Materials and Methods*). The 588-bp protected fragment indicates the presence of endogenous PADPRP mRNA. 36B4 represents the endogenous human ribosomal protein mRNA used as an internal control.

mine the levels of endogenous PADPRP RNA. Cells were induced with dexamethasone for 0, 24, or 48 h. Total RNA was then isolated and analyzed by the RPA, using a 588-bp probe homologous to the 3′ region of the endogenous PADPRP mRNA (*Materials and Methods*). In control cells **(Fig 2)**, there was no reduction in the endogenous levels of PADPRP RNA as determined by the equivalent levels of the 588-bp protected fragment from the labeled PADPRP probe. In antisense cells, a prominent 588/578-bp doublet was protected at time 0. This doublet was also observed in control cells at higher resolution, and may represent two different sized PADPRP transcripts in these cells. By 48 h the PADPRP mRNA doublet was almost undetectable in both antisense clones tested, even though there was no reduction in the level of the 220-bp control probe, indicating the selective elimination of the endogenous PADPRP transcripts.

Endogenous PADPRP Protein Is Depleted in Transfected Cells Stable clones of epidermal cells transfected with the antisense construct were treated with 1 μM dexamethasone and extracts containing equal amounts of protein separated by SDS-PAGE. Proteins were then immunoblotted, using polyclonal antiserum specific for human PADPRP. **Figure 3** shows the results of this experiment for one clone transfected with the PADPRP antisense construct, and one clone transfected with the vector alone. The levels of PADPRP were reduced by approximately 90% in antisense-containing cell lines by 48 h after treatment. In contrast, control cells showed no reduction in the level of PADPRP after induction with dexamethasone for as long as 72 h.

To confirm whether the reduction in immunologically detectable PADPRP represented a reduction in the level of endogenous PADPRP enzyme activity, measurements were performed using control or antisense cells treated with dexamethasone. PADPRP activity was noted to be only gradually reduced in control cells, so that by 48 h there was a 13% inhibition in activity. However, in antisense clones, induction by dexamethasone resulted in an 80% decrease in enzyme activity within 48 h **(Table I)**. Thus the levels

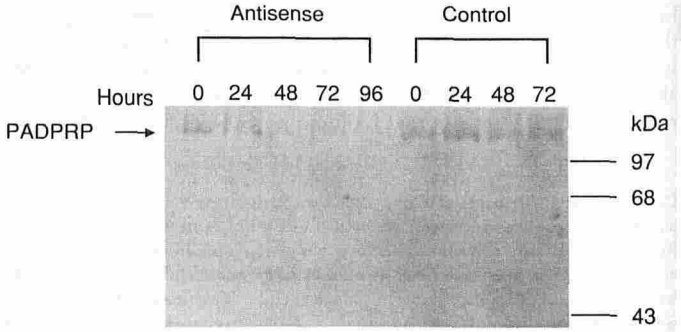

Figure 3. Dexamethasone induction of antisense RNA reduces cellular PADPRP protein content. Control and antisense-containing cell clones were grown in the absence or presence of dexamethasone (1 μM). At the times indicated, cells were collected and washed with PBS, and their protein concentration was determined. Equal amounts of total cellular protein (20 μg) were subjected to electrophoresis on duplicate SDS polyacrylamide gels. Enzyme-linked immunoblotting with rabbit antibodies to human PADPRP was performed as described in *Materials and Methods*. The 116-kDa *arrow* indicates the position of human PADPRP.

of endogenous PADPRP activity parallel the levels of immunologically detectable protein.

Human Keratinocytes with Antisense PADPRP Show a Reduced Response to DNA Damage Control or antisense cells were treated for 30 min with 400 μM MNNG to induce DNA strand breaks, either with or without a 48-h pretreatment with dexamethasone. Keratinocytes were then stained with rabbit antisera specific for PADPRP, as well as guinea pig antisera specific for poly(ADP-ribose) polymer. Double immunofluorescence of control cells revealed abundant levels of PADPRP either in the presence or absence of any combination of MNNG and dexamethasone **(Fig 4*A,D*)**, indicating that dexamethasone itself did not appreciably alter the levels of PADPRP in the keratinocytes. Staining for poly(ADP-ribose) polymer in control cells revealed that although very little polymer was formed in the absence of MNNG **(Fig 4*B,C*)**, treatment with MNNG resulted in a strong increase in the steady-state levels of polymer **(Fig 4*E,F*)**. When antisense cells were not pretreated with dexamethasone, the effect of MNNG on the levels of PADPRP and polymer in individual cell nuclei was identical to that of control cells (data not shown). However, when cells containing the PADPRP antisense construct are induced with dexamethasone prior to MNNG treatment, almost no PADPRP can be detected in individual cell nuclei, and the PADPRP that is present is localized to distinct focal regions within the nucleus **(Fig 4*G*)**. These same PADPRP-positive focal areas are also positive for poly(ADP-ribose) **(Fig 4*H,I*)**. However, the amount of polymer is drastically reduced compared to both control cells and dexamethasone-uninduced antisense cells.

Table I. PADPRP Activity Is Reduced in Antisense Keratinocytes[a]

Time (h) After Dexamethasone Induction	PADPRP Activity (pmoles NAD/min-mg protein)	
	Control	Antisense
0	40.5 ± 1.5	23.4 ± 1.2
24	40.0 ± 2.7	24.1 ± 0.8
48	35.1 ± 8.1	4.7 ± 1.5

[a] Keratinocytes harboring either constructs containing PADPRP antisense or vector alone were induced by dexamethasone for the indicated time periods. Cells were then sonicated, and extracts assayed for PADPRP activity as described previously [39]. Values represent an average of triplicates ± SD.

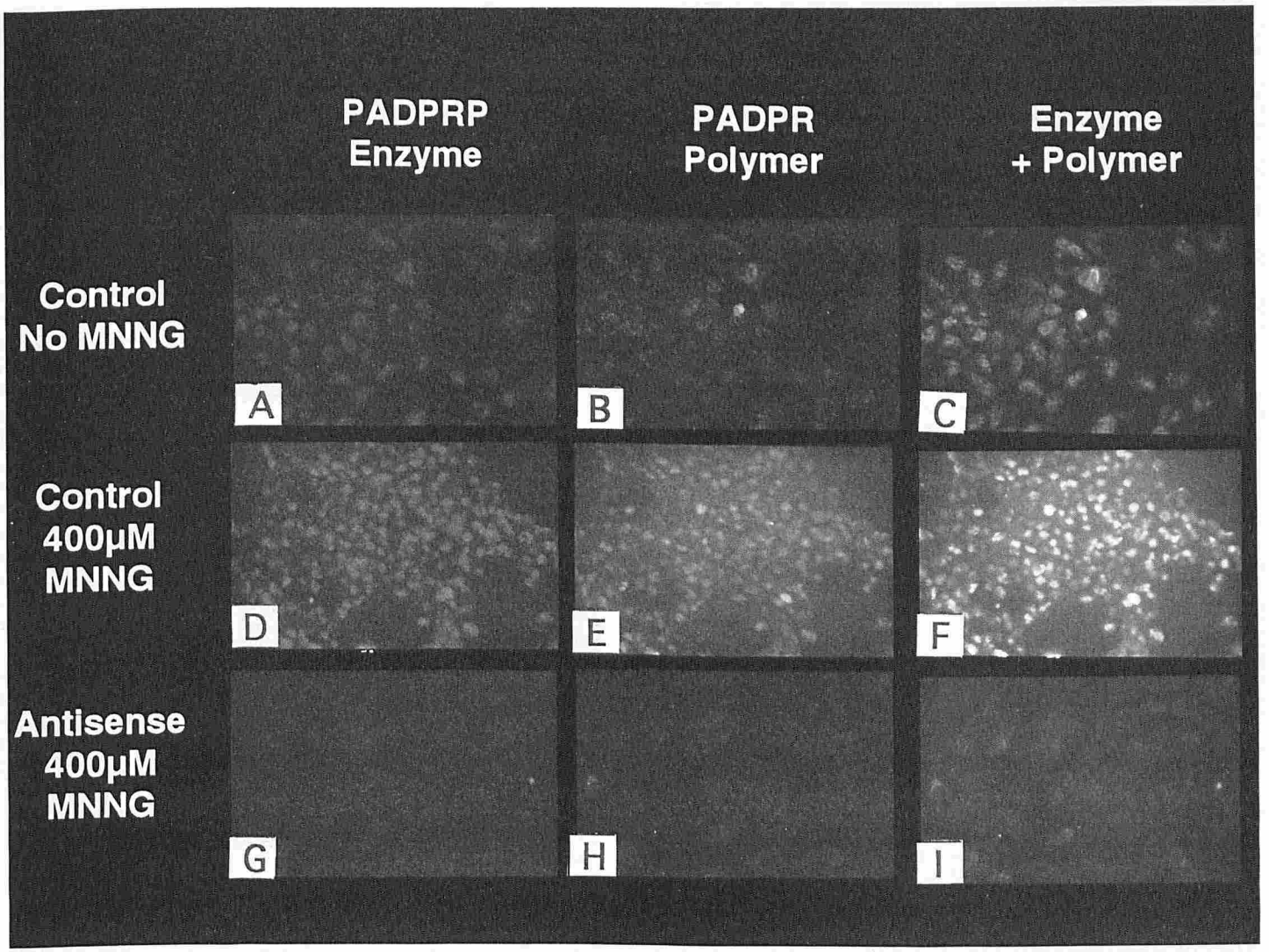

Figure 4. PADPRP antisense reduces response to keratinocytes to MNNG. Cells were induced with 1 μM dexamethasone 48 h prior to MNNG treatment. Control cells containing vector alone, *A–F*; antisense cells, *G–I*. Subsequently, the cells were analyzed by indirect immunofluorescence using the antibodies listed at the top of the figure (i.e., anti-PADPRP, *A, D, G*; anti-PADPRP polymer, *B, E, H*; anti-PADPRP plus anti-polymer, *C, F, I*), using either fluorescein or Texas red [25]. Nuclei stained yellow in *F* of the original double exposure, indicating the presence of both PADPRP and PADPR polymer. *Bar*, 0.1 mm for all panels.

Genetically Engineered Skin Cells Form Normal Epidermis in Grafts Keratinocytes derived from antisense or control clones were grown as a monolayer on tissue-culture plates, and then grafted onto the backs of nude mice, as described in *Materials and Methods*. After 3 weeks, one half of each graft was then fixed for histology, whereas the other half was quick frozen for immunocytochemistry. Both control and antisense keratinocytes formed an essentially normal epidermis as discerned by histologic analysis. When grafted keratinocytes and normal human forearm epidermis derived from punch biopsy were stained by hematoxylin and eosin, both tissues formed the compartments of the normal human epidermis including the basal layer, spinous layers, granular layers, and the anucleate cornified layers, demonstrating that these immortalized human keratinocytes can undergo terminal differentiation in the graft system (not shown). Frozen sections were stained immunocytochemically using antibodies specific for human keratin 10, which is expressed in the suprabasal layers of the epidermis, and for keratin K14, which is induced at the level of transcription in the lower layers of the normal human epidermis, but persists as a protein in all layers of the epidermis [25,32]. The reconstituted human epidermis demonstrates a similar staining pattern for human keratins 10 and 14 to that of normal human epidermis (not shown). Thus, the normal histologic and immunocytochemical pattern of differentiation is observed in the reconstituted grafted epidermis.

Antisense PADPRP RNA Is Inducible in Skin Grafts We then determined whether the antisense constructs are inducible in a reconstituted epidermis. Following grafting, animals were treated topically with dexamethasone. Grafts were quick frozen 0, 6, or 24 h after treatment and examined by *in situ* hybridization for the presence of antisense transcripts within the skin. Topical dexamethasone treatment for 24 h induced antisense transcripts within the epidermis of animals grafted with cells containing the antisense construct **(Fig 5*A,B*)**. Neither antisense cells that were not treated topically with dexamethasone, nor control cells that were either untreated or else treated with dexamethasone **(Fig 5*C,D*)**, showed hybridization to the antisense-specific riboprobe. As a positive control for the *in situ* hybridization technique, as well as to demonstrate that the epidermis within the graft site was derived from human cells, sections were hybridized with a riboprobe specific for the 3′ non-translated region of the mouse keratin 1 (MK1) RNA. This probe is homologous to mouse, but not human, epidermis [33,34]. The MK1 probe hybridizes only outside the periphery of the graft site, but not to the region where antisense

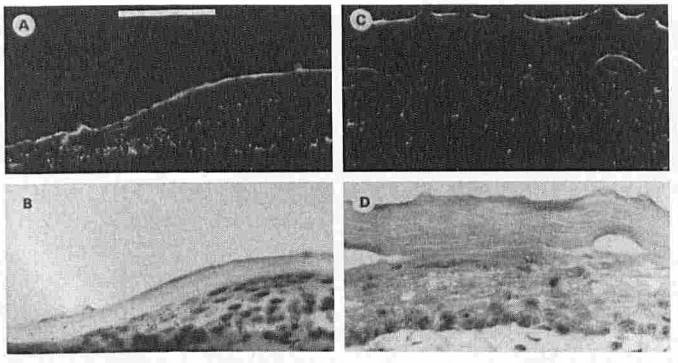

Figure 5. Topical dexamethasone induces PADPRP antisense transcripts *in vivo*. Cells transfected with antisense construct (*A,B*) or vector alone (*C,D*) were grafted onto nude mice. After 3 weeks, mice were treated topically with dexamethasone or acetone for 24 h. Frozen sections were derived and analyzed for antisense transcripts using a riboprobe complementary to antisense transcripts as described in *Materials and Methods*. *A*; darkfield microscopy of *B*. *C*; darkfield microscopy of *D*. *Bar*, 0.05 mm for all panels.

RNA was detected (not shown), demonstrating that the epidermis at the graft site is derived entirely from the grafted human keratinocytes.

PADPRP Polymer Is Induced *In Vivo* by Alkylating Agents

Finally, we determined whether poly(ADP-ribose) polymer formation, *via* its post-translational modification of nuclear proteins, could be induced in the grafted epidermis in response to DNA strand-breaking agents. Skin grafts were derived from the control human keratinocyte lines, and then treated with sulfur mustard by the vapor cup method. Staining of the skin sections with antibody specific for polymer demonstrated that, under the conditions used, polymer could be detected in sections of skin grafts treated with sulfur mustard, but not in control grafts **(Fig 6)**.

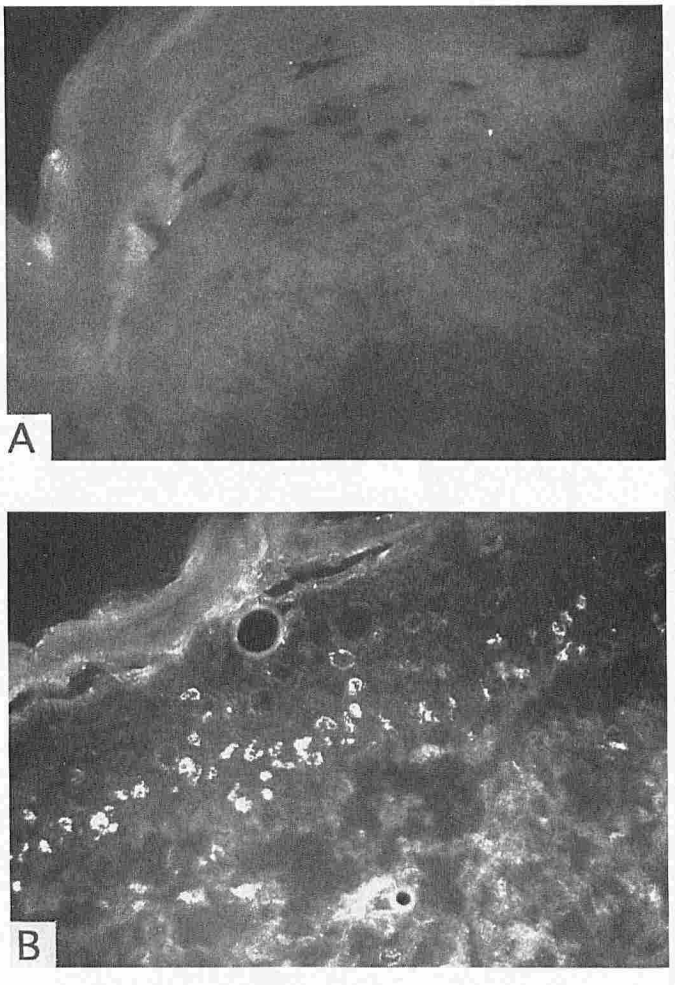

Figure 6. Topical sulfur mustard induces PADPRP polymer in reconstituted human epidermis. Grafted human epidermis was treated topically with sulfur mustard for 8 min, and the grafts embedded in OCT medium and quick-frozen. Sections of untreated (*A*) or treated (*B*) grafts were then analyzed by indirect immunofluorescence, using antibody specific for PADPRP polymer (*Materials and Methods*). *Bar*, 0.05 mm.

DISCUSSION

PADPRP is implicated in several biologic processes including DNA repair, replication, and cellular differentiation, yet none of these roles have been firmly established. The importance of the above processes in the skin led us to develop a strategy for the selective inhibition of PADPRP in human keratinocytes to analyze the various biologic parameters in cells with greatly reduced levels of this normally abundant nuclear enzyme.

The stably transfected lines of human keratinocytes displayed a dexamethasone-dependent synthesis of an RNA complementary to the mRNA transcribed from the PADPRP gene. This resulted in the selective lowering of endogenous PADPRP mRNA, protein, and enzyme activity in culture. All of the antisense and control cell clones formed a histologically and immunocytochemically normal epidermis when grafted onto nude mice. In our study some of the cell lines gave rise to skin lesions resembling carcinomas, as was the case when we grafted human keratinocytes immortalized with SV40 large T antigen (data not shown). In other cases, no human skin structure was found within the graft site, although some cell lines formed nests of cells beneath the surface of the graft site. Of five keratinocyte lines tested, only one formed a normal epidermis. There have been few other reports in which immortalized human keratinocytes have been successfully grafted onto nude mice. In one other case, spontaneously transformed human keratinocytes formed a morphologically normal epidermis in grafts on nude mice [31]. However, it has been recently determined that this cell line carries a mutant tumor suppressor gene p53 [35] and may therefore have only limited value for further studies.

Because these keratinocytes can be used as a recipient for plasmid constructs that lower PADPRP activity *in vitro*, and form a structure that is morphologically indistinguishable from normal human epidermis, they will be useful in testing the role of PADPRP in such processes as the modulation of the skin response to DNA damaging agents, and in epidermal differentiation. We have demonstrated the effectiveness of the antisense constructs in lowering the levels of PADPRP in culture. Additionally, we have shown that topical administration of dexamethasone, as well as intravenous administration (data not shown), induces the transcription of antisense PADPRP RNA. Thus, there is a good probability that endogenous PADPRP levels will be lowered *in vivo* as well.

Several features of this engineered epidermis make it extremely useful for further long-term studies of PADPRP or other enzymes within the epidermis. In addition to the aforementioned ability of these cells to form a morphologically and immunocytochemically normal epidermis, we have been able to immediately monitor the level of DNA damage within the epidermis *in vivo* by the use of monospecific antibodies directed against the poly(ADP-ribose) polymer. Thus, we can manipulate antisense, and measure the immediate response to DNA damage, as well as the long-term response, such as the mutation of genes critical for epidermal differentiation or transformation.

The poly(ADP-ribosyl)ation of a number of proteins involved in DNA metabolism and gene expression is no doubt important in the above processes. In cultured keratinocytes, expression of PADPRP antisense drastically altered the pattern of poly(ADP-ribosyl)ation **(Fig 4)**. We would therefore expect profound effects on the

biology, biochemistry, and chromatin structure of these cells. Previous studies with PADPRP antisense RNA showed that upon depletion of PADPRP levels by this method, there was an ambient decrease in DNA strand-break repair rejoining, a change in preferential repair of the DHFR gene [16], and increase in the amplification of genes [17]. There was also a decrease in cell survival to alkylating agents [15]. In addition, we would expect there to be large changes in the ability of these cells to differentiate in culture and in the reconstructed epidermis. This is evidenced by our previous study in which we expressed PADPRP antisense RNA in 3T3 cells and prevented differentiation from preadipocytes to adipocytes [21]. In this case, it appears that PADPRP is important in the terminal replicative cycles prior to differentiation [21]. In cultured keratinocytes, a similar phenomenon of a near-synchronous wave of replication prior to differentiation is observed. Various models, all of which involve chromatin restructuring, have been proposed to explain the necessity for DNA replication prior to terminal differentiation [36]. Implicit in many of these models is the repositioning or alteration of nucleosomes, which may result in the activation or inhibition of specific genes and may involve various nuclear protein modifications, including poly(ADP-ribosyl)ation. In this regard, poly(ADP-ribosyl)ation may aid in either relaxation [37] or condensation of chromosomal proteins around the replicating regions of chromatin [38]. Whether PADPRP function is essential to the terminal differentiation process in keratinocytes can now be tested directly with the *in situ* system developed in this study.

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